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(57) Abstract

A method for the quantitative determination of human acute phase serum amyloid A protein (A-SAA) comprises contacting a sample of a biological fluid with antibody specific for A-SAA, the sample being reacted with an organic solvent prior to or simultaneous with antibody contact. The organic solvent is suitably a C_1-C_4 alcohol and the antibody can be used on the solid phase and as a component of the detection system of an enzyme linked immunosorbant assay. The method provides a sensitive and reliable measure of A-SAA and inflammatory status which can be used for the diagnosis and clinical management of both acute and chronic inflammatory conditions.

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Description

METHOD FOR THE QUANTITATIVE MEASUREMENT OF HUMAN ACUTE PHASE SERUM AMYLOID A PROTEIN; RECOMBINANT PROTEIN; SPECIFIIC ANTIBODY

Technical Field

This invention relates to a method for the quantitative determination of human acute phase serum amyloid A protein (A-SAA) which distinguishes between the A-SAA and constitutive (C-SAA) forms of SAA.

Background Art

The mammalian acute phase response is the first line of systemic defence elicited by stimuli such as infection, trauma, myocardial infarction, neoplasms, and surgery. It is initiated and maintained by a large number of pro-inflammatory mediators including cytokines, glucocorticosteroids and anaphylatoxins and involves a wide range of complex physiological changes including elevated circulating concentrations of hepatically synthesised acute phase reactants (APRs). In man, this latter class includes the "major" APRs, serum amyloid A (SAA) and C-reactive protein (CRP) (reviewed by Steel, D.M. and Whitehead, A.S. (1994) Immunol. Today (England) *15*, 81).

The human SAA gene family is comprised of four known genes that have been localised to the short arm of chromosome 11p15.1. The *SAA1* and *SAA2* genes specify the two acute phase SAA proteins A-SAA1 and A-SAA2 respectively which are both 104 amino acid, 12.5 kDa proteins that share 93% amino acid sequence identity. A number of allelic forms have been identified by amino acid sequence analysis of A-SAA isolated from plasma. The A-SAA1 protein has three allelic forms whereas the A-SAA2 protein has two. A third gene *SAA3* which shows 71% nucleotide identity with *SAA1* and *SAA2* is a pseudogene. Constitutive SAA (C-SAA) is the third expressed SAA family member and is the product of the *SAA4* gene. C-SAA levels

characteristically do not increase as a result of inflammation and exist in serum at concentrations between 80-140 mg/L (Yamada, T. *et al.* (1994) Int. J. Exp. Clin. Invest.. *1*, 114). C-SAA differs from A-SAA with respect to peptide length, being eight amino acids longer, and shares only 55% identity with A-SAAs. Additionally, C-SAA may be post-translationally modified by glycosylation at a single site. In common with the A-SAAs, C-SAA rapidly associates with high density lipoprotein (HDL3) when released into the circulation.

Circulating concentrations of A-SAA can increase up to 1000 mg/L within 24-48 hours of an acute stimulus (Marhaug, G. (1983); Scand. J. Immunol. *18*, 329) indicating an important protective role for these proteins; however, no definitive function has been demonstrated for the A-SAA proteins. Recent studies variously suggest that A-SAA has chemoattractant activity, may play a role in lipid metabolism and immunosuppression and may inhibit the oxidative burst in neutrophils.

During chronic inflammation A-SAA levels remain significantly elevated reflecting the continued persistence of underlying pathological inflammatory processes that can contribute to long term tissue damage. An occasional consequence of chronic inflammation is reactive secondary amyloidosis, a progressive fatal condition in which amyloid A protein (AA), a cleavage product of A-SAA, is the major component of insoluble fibrous deposits that accumulate in major organs. The sustained elevation of A-SAA in chronic inflammatory conditions suggests that A-SAA is an important indicator of disease status. However, the measurement of A-SAA concentration has not been used for routine clinical diagnosis and clinical management, due in part to the difficulty in raising specific antisera against human A-SAA (Pepys, M.B. *et al.* (1984); British Medical Journal *288*, 859).

Several methods, however, have been reported for the measurement of SAA levels: these include (i) radioimmunoassays and single radial immunodiffusion procedures (Chambers, R.E. and Whicher, J.T. (1983); J. Immunol. Methods *59*, 95; Marhaug, G.

(1983) *supra*; Taktak, Y.S. and Lee, M.A. (1991); J. Immunol. Methods *136*, 11); (ii) ELISA based assays (Zuckerman, S.H. and Suprenant, Y.M.(1986); J. Immunol. Methods *92*, 37-43; Dubois, D.Y. and Malmendier, C.L. (1988); J. Immunol. Methods *112*, 71-75; Sipe, J.D. *et al.* (1989); J. Immunol. Methods *125*, 125-135; Yamada, T. *et al.* (1989); Clin. Chim. Acta *179*, 169-176; Tino-Casl, M. and Grubb, A. (1993); Arm. Clin. Biochem *30*, 278-286); (iii) nephelometric methods (Vermeer, H. *et al.* (1990); Clin. Chem *36*, 1192; Yamada, T. *et al.* (1993); Ann. Clin. Biochem. *30*, 72-76); (iv) an electrophoretic procedure (Godenir, N.L. *et al.* (1985); J. Immunol. Methods *83*, 217); (v) an immunochemiluminescence procedure (Hachem, H. *et al.* (1991); Clin. Biochem *24*, 143-147); (vi) an automated method based on a monoclonal-polyclonal antibody solid phase enzymeimmunoassay (Wilkins, J.W. *et al.* (1994); Clin. Chem *40*(7), 1284-1290); and (vii) time-resolved fluorometric immunoassay (Malle, E., *et al.* (1995); J. Immunol. Methods *182*, 131). As SAA in serum exists as one of the apolipoproteins associated with HDL3 particles many of these methods require denaturation of the serum samples (in an effort to eliminate the masking effect previously observed to be a problem in the accurate quantification of SAA) prior to carrying out the assay. Many assays previously reported have either measured total SAA or have been based on anti-sera raised against total SAA and have not been documented as being able to distinguish between the A-SAA and C-SAA proteins. Furthermore, many of these assays require an overnight incubation.

Problems have been encountered obtaining a soluble purified native A-SAA: purification of A-SAA protein from large volumes of blood is characterised by poor yields (Godenir, N.L. *et al.* (1985) *supra*), limited solubility (Bausserman, L.L. *et al.* (1983); J. Biol. Chem. *258*, 10681) and the heterogeneous nature of the A-SAA recovered. In addition, A-SAA purified from serum may contain trace amounts of other serum components thereby potentially compromising studies of A-SAA function that involve sensitive bioassays.

There is a need for a method which provides a sensitive and reliable measure of A-SAA and inflammatory status which can be used

for the diagnosis and clinical management of both acute and chronic inflammatory conditions.

Disclosure of Invention

The invention provides a method for the quantitative determination of human acute phase serum amyloid A protein (A-SAA), which comprises contacting a sample of a biological fluid with antibody specific for A-SAA, said sample being reacted with an organic solvent prior to or simultaneous with antibody contact.

By biological fluid herein is meant body fluids such as plasma, serum, synovial fluid, urine and bile, more especially plasma and serum, a perfusate, tissue support media or cell/tissue culture media.

The biological fluid can be diluted.

Preferably, the antigen used to raise the anti-A-SAA is recombinant A-SAA.

Recombinant protein technology offers a means of generating a reliable, renewable, homogeneous source of A-SAA.

Most preferably the antigen used to raise the anti-A-SAA is recombinant A-SAA2.

No other immunoassay has been reported which utilises antibodies raised against recombinant A-SAA2.

The production of recombinant human A-SAA2 in *E.coli* as a GST fusion protein using the pGEX expression system (Smith, D.B. and Johnson, K.S. (1988); Gene *67*, 447) is described in Example 1. Expression of A-SAA2 in this system permits the recovery of soluble recombinant A-SAA2 protein following thrombin cleavage of the fusion protein when used in the presence of a mild non-ionic detergent as hereinafter described.

Preferably, the organic solvent is a polar organic solvent.

More especially, the organic solvent is a C_1-C_4 alcohol.

The organic solvent can include an amount of a C_1-C_4 ether.

Preferably, the organic solvent is used in an amount of 10-50% v/v of the sample diluent.

Most preferably, the organic solvent is used in an amount of 20-30% v/v of the sample diluent.

In a preferred embodiment, the antibody is used on the solid phase and as a component of the detection system of an enzyme linked immunosorbant assay (ELISA).

Antibodies raised against recombinant A-SAA2 were found to be specific for the A-SAAs and were used to develop a sandwich ELISA to quantify A-SAA levels in serum, as hereinafter described.

The ELISA described herein has a high sensitivity and can detect A-SAA concentrations of 5µg/L in human serum and tissue culture media.

The invention also provides a test kit or pack for carrying out the method according to the invention.

The invention also provides substantially pure recombinant A-SAA2.

Further the invention provides antibodies specific for A-SAA1 and A-SAA2, more especially IgG class antibody, most especially polyclonal IgG.

The invention provides antibodies specific for the acute phase SAAs, A-SAA1 and A-SAA2, which show no cross-reactivity with the constitutively expressed SAA, namely C-SAA or other acute phase protein.

Brief Description of Drawings

Fig. 1 is a photograph of an SDS-PAGE gel following analysis of recombinant A-SAA2 expressed from pGEX as described in Example 2;

Fig. 2 is a photograph of an SDS-PAGE gel following analysis of extracted recombinant GST-(A-SAA2) fusion protein as described in Example 2;

Fig. 3 is a photograph of an immunoblot following analysis of antiserum raised against recombinant A-SAA2 when tested for reactivity against recombinant and native A-SAA and potential cross-reactivity *versus* C-SAA as described in Example 3;

Fig. 4 is a standard curve for A-SAA for the assay described in Example 4;

Fig. 5 is a graph of SAA concentration (μg/L) *versus* time (hours) for two samples as described in Example 5;

Fig. 6 is a graph of absorbance at 450nm *versus* A-SAA concentration (μg/L) for various concentrations of an organic solvent in a sample dilution buffer as described in Example 6;

Fig. 7 is a graph of optical density (absorbance) at 450nm *versus* serial dilution for various samples as described in Example 9; and

Fig. 8 is a bar graph representation of A-SAA concentration in the serum of rheumatoid arthritis patients as described in Example 10.

Modes for carrying out the invention

The invention will be further illustrated by the following Examples.

Example 1

Construction of the A-SAA2 protein expression vector

The coding region of A-SAA2 was amplified from the A-SAA2 cDNA clone by the polymerase chain reaction (PCR) with the concomitant introduction of sequence specifying an additional glycine residue and a *Bam*H1 restriction site at the 5' end (oligonucleotide primer sequence 5'-CGGGATCCGGGCGAAGCTTCTTTT CGTTC-3' (SEQ ID NO.1)) and an *Eco*R1 site at the 3' end (oligonucleotide primer sequence 5'-CGGAATTCAGTATTTCTCAGGCAGGCC-3' (SEQ ID NO.2)). The PCR product was digested with BamHI and EcoRI, gel purified, and ligated into the Glutathione S-Transferase (GST) fusion protein expression vector pGEX-2T (Pharmacia Fine Chemicals, Milton Keynes, U.K.). The A-SAA2 coding region was inserted in frame into the pGEX-2T vector to produce a construct in which A-SAA2 expression was under the control of the isopropyl ß-D-thiogalactosidase (IPTG) inducible *tac* promoter and GST ribosome binding site. DNA sequence analysis of the resulting pGEX-(A-SAA2) confirmed that it carried the entire unmodified A-SAA2 coding region positioned downstream of the GST coding region with no mutations resulting from the PCR process.

Example 2

Induction of *E. coli* cultures for high level expression of recombinant A-SAA2 protein

Plasmid pGEX-(A-SAA2) was transformed into the *E. coli* expression strain NM554. Transformants were isolated and grown overnight at 37°C. Overnight cultures were diluted 1/100 in Luria

broth containing 100 µg/ml ampicillin (Boehringer Mannheim, East Sussex, U.K.) and grown to an OD_{600} value of 1.0. Expression of recombinant fusion protein was induced in culture with 0.1 mM isopropyl ß-D-thiogalactosidase (IPTG: Sigma, Dorset, U.K.) for 5 h at 37°C. Cultures were centrifuged at 5000 x g for 10 min at 4°C. Upon induction a 38.5 kDa GST-(A-SAA2) fusion protein was produced (see Fig. 1, lane 4) constituting approximately 5% of the total cellular protein.

Fig. 1 illustrates the expression of the recombinant A-SAA2 protein from pGEX(A-SAA2) analysed by SDS-PAGE.

Key to Fig.1:

Lane 1: protein molecular weight markers 55.6, 39.2, 26.6, 12.5, 6.5 kDa;

Lane 2: pGEX(A-SAA2) uninduced;

Lane 3: pGEX-2T expression vector induced with IPTG;

Lane 4: pGEX(A-SAA2) induced with IPTG;

Lane 5: thrombin cleaved mature A-SAA2 product; and

Lane 6: purified recombinant A-SAA2 following ion-exchange chromatography.

Cell pellets were resuspended in 1/50 of the starting volume in lysis buffer [PBS pH 7.3 (Gibco/BRL, Paisley, U.K.) containing 0.2 mg/ml lysozyme (Sigma); 5 mM EDTA (BDH, Merck, Dorset, England); 0.1% (v/v) Triton X-100 (BDH); 50 mM benzamidine (Sigma); 0.1 mM PMSF (Sigma) and 0.5 mg/ml iodoacetamide (Sigma)] and incubated for 1 h at room temperature. Solubilisation

of GST-(A-SAA2) from lysed *E.coli* cell pellets requires the presence of 0.1% (v/v) Triton X-100 (see Fig. 2).

Recombinant GST-(A-SAA2) fusion protein was tested for solubility in the presence and absence of nonionic detergent 0.1% (v/v) Triton X-100 followed by SDS-PAGE analysis. The results are depicted in Fig. 2.

Key to Fig. 2:

Lane 1: protein molecular weight markers 55.6, 39.2, 26.6, 12.5 kDa;

Lane 2; Insoluble fraction after cell lysis without the presence of 0.1% (v/v) Triton X-100;

Lane 3: Soluble fraction without the presence of 0.1% (v/v) Triton X-100;

Lane 4; Insoluble fraction after cell lysis in the presence of 0.1% (v/v) Triton X-100; and

Lane 5: Soluble fraction in presence of 0.1% (v/v) Triton X-100.

Lysates were sonicated on ice (3 x 20 second bursts) to obtain complete lysis, centrifuged at 10000 x g for 10 min at 4°C and filtered through a 0.45 µM Millipore (Millipore is a trade mark) filter to remove particulate material. Clarified sonicates were passed through a Glutathione Sepharose 4B column (Pharmacia) to which the GST-(A-SAA2) fusion protein bound. Contaminating *E. coli* proteins were removed by washing with ten column volumes of PBS (pH 7.3), and the recombinant A-SAA2 protein was directly cleaved from the GST moiety on the Glutathione Sepharose 4B column using thrombin (Sigma) (5 U/mg protein bound) in PBS (pH 7.3) 0.1% (v/v) Triton X-100 at room temperature for 6 h. Cleavage by thrombin in the

presence of 0.1% (v/v) Triton X-100 yielded a soluble A-SAA2 product of 12.5 kDa [the predicted size for mature A-SAA2] (see Figs. 1 (cleaved) and 2 (uncleaved), lane 5). The column eluate containing recombinant A-SAA2 was collected and stored at 4°C. The recombinant A-SAA2 sample was further purified by ion exchange chromatography using a column of high performance Sepharose Q (Pharmacia) equilibrated with 0.1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 10.0) and eluted with 0.1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 10.0), 0.1 M NaCl. Fractions were collected and analysed by SDS-PAGE.

Further purification of recombinant A-SAA2 was achieved using ion exchange chromatography and the resulting protein could be resolved as a single band on SDS-PAGE (Fig. 1, lane 6). N-terminal amino acid sequence of the 12.5 kDa product was Gly-Ser-Gly-Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Glu-Ala-Phe-Asp-Gly-Ala-Arg-Asp (SEQ ID NO.3), confirming its identity as A-SAA2 with an amino terminal Gly-Ser-Gly extension derived from the fusion protein. The N-terminal sequencing of recombinant A-SAA2 was carried out by electroblotting the recombinant A-SAA2 onto a ProBlott (ProBlott is a trade mark) membrane and staining with amido black prior to N-terminal amino acid sequencing on a Biosystems model 473A protein sequencer. Approximately 3 mg of recombinant A-SAA2 was obtained *per* litre of bacterial culture.

Fractions following purification on the affinity column were also analysed by immunoblotting. For the SDS-PAGE and the immunoblotting anti(A-SAA) antiserum (see Example 3) was used with peroxidase-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody. The protein content of the fractions was determined using bicinchoninic acid solution (Sigma) with crystalline bovine serum albumin (Sigma) as standard. Recombinant A-SAA2 was stored at 4°C in buffer A (20 mM Tris-HCl (pH 8.4), 150 mM NaCl and 0.1% (v/v) Triton X-100).

Example 3

Antibodies to A-SAA2

Rabbits were immunised intramuscularly with recombinant A-SAA2 purified from SDS-PAGE gels prepared in Example 2, according to the method of (Hager, D.A. and Burgess, R.R. *et al.* (1980); Anal. Biochem. *109*, 76) as follows: Day 1, 1ml of 1 mg/ml recombinant A-SAA2 in Freunds complete adjuvant (Sigma); Days 14 and 21, 1 ml of 1 mg/ml recombinant A-SAA2 in Freunds incomplete adjuvant (Sigma). Blood was drawn on Day 28. IgG-anti(A-SAA) was isolated by affinity chromatography on immobilised Protein A (Pharmacia). The resulting antiserum was tested for cross-reactivity with other human SAA protein family members and serum components by immunoblot analysis (see Fig. 3).

Key to Fig. 3:

Lane 1: recombinant A-SAA2;

Lane 2: Serum of an acute phase patient;

Lane 3: Non-acute phase serum;

Lane 4: NIBSC (National Institute of Biological Standards and Controls) A-SAA;

Lane 5: recombinant C-SAA;

Lane 6: recombinant A-SAA1; and

Lane 7: recombinant A-SAA2 spiked into non-acute phase serum.

The antiserum reacted with (i) purified recombinant A-SAA2 (Fig. 3, lane 1); (ii) A-SAA (but no other molecular species) present

in the serum of a patient with inflammation (Fig. 3, lane 2) and A-SAA obtained from the NIBSC (Fig. 3, lane 4); (iii) recombinant A-SAA2 spiked into non-acute phase serum (Fig. 3, lane 7) and (iv) recombinant A-SAA1 [expressed and purified as for A-SAA2] (Fig. 3, lane 6). Antibodies raised against recombinant A-SAA2 generate equivalent signals with both recombinant A-SAA1 and recombinant A-SAA2 in the immunoblot (compare lanes 1 and 6 of Fig. 3) indicating that the binding capacity for each isoform is essentially equivalent. In addition, the antibodies raised against recombinant A-SAA2 did not cross react with purified C-SAA [expressed and purified as for A-SAA2] (Fig. 3, lane 5) or any component of non-acute phase serum (Fig. 3, lane 3).

Example 4

An ELISA procedure in accordance with the invention involves coating of microtitre plates with purified IgG[anti-A-SAA] obtained as described in Example 3 and performance of the assay procedure:

1. Coating of microtitre plates

Microtitre maxisorp plates (Nunc, Denmark) were coated with affinity purified IgG[antiSAA2] (1.0µg/ml in 0.1M carbonate buffer, pH 9.6) overnight at 4°C. Plates were washed twice with PBS containing Tween-20 0.05 % (PBST), and a BSA-containing blocking buffer was added to the wells.

Microtitre plates were incubated for 1 hour at 37°C. The blocking buffer was removed and the plates were dried overnight at 37°C. The microtitre plates were sealed and stored at 4°C until required.

2. Assay procedure

(a) Serum samples and assay calibrator (recombinant A-SAA) were diluted in the sample dilution buffer:

20mM Tris-HCl pH 7.8

150mM NaCl

25% (v/v) propan-2-ol

and 100µl of each dilution were added in duplicate to the microwells. After incubation at room temperature (20-25°C) for 60 minutes with uniform shaking, wells were washed four times with 350µl PBST using a plate washer.

(b) Enzyme conjugate (IgG[anti-SAA2]-HRP) was diluted in conjugate dilution buffer (50mM TrisHCl pH 7.8, 150mM NaCl, 1% (w/v) BSA) and 100µl aliquots were added to the wells. The enzyme conjugate was produced essentially as described by Duncan, R.J.S. *et al.* (1983); Anal. Biochem. *132*, 68. HRP was obtained from Biozyme Ltd., UK. Microtitre plates were incubated at room temperature (20-25°C) for a further 60 minutes with uniform shaking. The wells were washed four times with 350µl PBST.

(c) 100µl of stabilised tetramethylbenzidene (TMB) substrate was added to each well using a multichannel pipette, and plates were incubated at room temperature for 15 minutes. Colour development was stopped using 100µl IN H_2SO_4 and plates were read immediately at 0.D.450nm on a plate reader.

(d) Standard Curve for A-SAA: Native SAA obtained from the National Institute of Biological Standards and Controls (NIBSC, UK) and recombinant SAA2 protein were both used to generate standard curves. Results were identical in both cases. The A-SAA standard curve range of 5-750µg/L is prepared in sample dilution buffer as described above (20mM Tris-HCl pH 7.8, 150mM NaCl, 25% (v/v) propan-2 ol). Each of the specified parameters of temperature, time, and concentration may be varied in accordance with standard laboratory practise without substantially affecting the utility of the procedure. The

standard curve obtained using the SAA standards was used to calculate the SAA concentration in the test samples (Fig. 4).

Example 5

The use of propan-2-ol in the sample dilution buffer was tested for suitability as a routine reagent. Samples were diluted in propan-2-ol dilution buffer and left at room temperature for a period of 24 hours. The samples were assayed at different time points; immediately after dilution and subsequently at 0.5 hour, 1 hour, 3 hours, 6 hours and 24 hours, to investgate if any change in immunoreactivity occurred when samples were left in the organic sample dilution buffer. No variability of signal return was observed for the different serum samples indicating that there is no degradation of A-SAA epitopes and that the propan-2-ol dilution buffer can be used routinely in clinical investigations. The results are depicted in Fig. 5.

Example 6

Masking effect

Spiking experiments to investigate if any serum proteins interfere with A-SAA quantitation were carried out. In these experiments we examined if we could recover the same amount of A-SAA following the spiking of known amounts of recombinant A-SAA into non-acute phase serum. While 100% detection was observed when the recombinant A-SAA was spiked into sample dilution buffer, only 26% detection was obtained following the spiking of recombinant SAA2 into non-acute phase serum as shown in Table 1.

Table 1

The % recovery using the ELISA following spiking of recombinant A-SAA into non-acute phase serum.

Dilution buffer	recombinant A-SAA2 µg/L spiked	recombinant A-SAA2 µg/L recovered	% recovery
-	750	190	26
	375	120	32
	188	40	21

This shows that certain serum components mask the SAA signal. Tino-Casl & Grubb (1993) also observed masking of the SAA signal and theorised that IgG and HDL were the dominant proteins in the masking fractions. They proposed that there was no detectable masking of the antigen signal in their ELISA procedure if samples were prediluted 1:500 prior to assay and a final concentration of 0.2% (v/v) normal serum was present in their dilution buffer. However no spiking experiments to examine the absolute level of initial or residual masking were reported in their study. In our assay system, 0.2% serum in the dilution buffer did not reduce the masking effect to any great extent (data not shown). A variety of buffers containing various reagents such as Tween 20, Triton X-100, Nonidet P-40, SDS, urea did not reduce or eliminate the masking effect on the SAA signal as shown in Table 2.

Table 2

The ELISA was used to investigate if different detergents/denaturants in the sample dilution buffer could increase the recovery of spiked recombinant A-SAA in nonacute phase serum.

Dilution buffer	recombinant A-SAA2 µg/L spiked	recombinant A-SAA2 µg/L recovered	% recovery
	750	190	26
2M Urea	750	188	25
6M Urea	750	210	28
8M Urea	750	190	26
1% Triton	750	180	24
1% Triton +1% BSA	750	150	20
1% Tween	750	202	27
1% Tween + 1% BSA	750	202	27
1% SDS	750	210	28
1% SDS + 1% BSA	750	190	26
1% NP40	750	173	23
1% NP40 + 1% BSA	750	158	21

It was observed that the presence of propan-2-ol at a concentration of 25%(v/v) as well as other alcohols/organic solvents in the sample dilution buffer led to the complete quantitative recovery of the A-SAA signal following spiking of non-acute phase serum with recombinant A-SAA as shown in Table 3 and Fig. 5.

Table 3

The ELISA was used to investigate if different organic solvents in the sample dilution buffer could increase the recovery of spiked recombinant A-SAA2 in non-acute phase serum.

Dilution buffer	recombinant A-SAA2 µg/L spiked	recombinant A-SAA2 µg/L recovered	% recovery
No organic solvent	750	190	26
25%Propan-2-ol	750	733	98
25% Methanol	750	713	95
25% Ethanol	750	720	96
25% Ethanol/Ether 3:1	750	730	97

Fig. 5: Fig. 5 depicts the results of an investigation of the unmasking capacity of various concentrations of propan-2-ol in the sample dilution buffer. Recombinant A-SAA was spiked into non-acute phase serum and assayed in dilution buffer containing no propan-2-ol.

Key to Fig. 5:

(A) Recombinant A-SAA2 was spiked into buffer A and assayed in dilution buffer containing no propan-2-ol

(B) Recombinant A-SAA2- was spiked into non acute phase serum and assayed in dilution buffer containing:

(C) 10 % propan-2-ol;

(D) 20 % propan-2-ol;

(E) 25 % propan-2-ol; and

(F) 40% propan-2-ol.

It was also observed that the presence of 25%(v/v) propan-2-ol in the dilution buffer when preparing the native SAA standard (NIBSC) increased the signal obtained, thus demonstrating that purified native SAA spiked into serum is also subject to masking by serum components as shown in Example 7.

Example 7

Comparative Data

Comparison between recombinant SAA2 and serum samples containing an identical amount of acute phase SAA is essential to ensure that the IgG [anti-SAA2 (recombinant)] recognises both types in a similar manner. As can be seen from Table 4, this is indeed the case whereby IgG raised against the recombinant SAA2 reacts identically with acute phase SAA purified from human serum.

Table 4

Comparative reactivity between recombinant and native SAA with IgG raised against the recombinant form of SAA2.

[SAA] (µg/L)	$A_{450/630nm}$ Recombinant SAA2	Native SAA
1500	1.492	1.217
750	0.807	0.915
325	0.467	0.604
187	0.213	0.311
94	0.086	0.139
46	0.046	0.087
23	0.033	0.043
0	0.006	0.000

Example 8

Clinical Utility

The upper limit of normal for acute phase SAA in serum is known to be less than 10mg/L. Using the immunoassay described in Example 4, no detectable amounts of acute phase SAA were detectable in many normal human serum samples and less than 10mg/L was detectable in

SAA are elevated in the case of both rheumatoid arthritis and acute pancreatitis, respectively as measured by the enzyme immunoassay.

Table 5

SAA levels in diseases states as measured by the enzyme immunoassay.

Disease State	[SAA] (mg/L, mean ± SD)	n
Rheumatoid Arthritis	92.4 ± 150	19
Acute Pancreatitis	309 ± 140	15

Example 9

Some previous reports of methods to measure A-SAA in patient serum have identified problems associated with quenching of the A-SAA signal by serum components (Casl *et al.*, 1993). To address this issue, we conducted experiments in which purified recombinant A-SAA2 was added to serum (spiked) and interference caused by serum components was quantified. Recombinant A-SAA2 at a known concentration was spiked into (i) buffer A and (ii) non acute phase serum, and assayed using the sample dilution buffer without 25% (v/v) propan-2-ol. Only 26% recovery of signal was observed following the spiking of recombinant A-SAA2 into non-acute phase serum as shown in Table 1.

The above spiking experiment was repeated using sample dilution buffer containing 25% (v/v) propan-2-ol. In the presence of the organic solvent almost complete recovery of signal was observed following spiking of recombinant A-SAA2 into non-acute phase serum (Table 6).

Table 6

Spiking of recombinant A-SAA2 into non-acute phase serum. Serial dilutions were carried out in dilution buffer with 25% (v/v) propan-2-ol.

Dilution buffer with 25%(v/v) propan-2-ol	recombinant A-SAA2 µg/L spiked	recombinant A-SAA2 µg/L recovered	% recovery
	750	740	98
	375	358	95
	188	178	95

Although not wishing to be bound by any theoretical explanation of the invention, the propan-2-ol sample dilution buffer most likely achieves signal recovery by disrupting the hydrophobic apolipoprotein complexes thereby facilitating antibody access to otherwise hidden A-SAA epitopes. From our studies purified recombinant with A-SAA2 in buffer A, A-SAA2 spiked into non-acute phase serum, and native A-SAA in serum from a rheumatoid arthritis patient, all show similar serial dilution profiles when diluted in our sample dilution buffer (Fig. 7). The presence of propan-2-ol in the sample dilution buffer offers a simple, rapid alternative to previous methods used to unmask the A-SAA in serum samples prior to immobilisation on solid phases.

Example 10

Serum Samples

As in further exemplification of the clinical data of Example 8, normal serum samples were obtained from blood donors aged 18-65 years. Rheumatoid arthritis serum samples were obtained from patients undergoing routine assessment in the Rheumatology Clinic of St James Hospital, Dublin. Samples were stored at -20°C prior to use.

patients undergoing routine assessment in the Rheumatology Clinic of St James Hospital, Dublin. Samples were stored at -20°C prior to use.

In the A-SAA sandwich ELISA serum samples were routinely run at 1/200 dilution. The lower and upper limits of the low range standard curve were 5 µg/L and 100 µg/L respectively. The lower and upper limits of the high range standard curve were 50 µg/L and 750 µg/L, respectively (Fig. 4), and samples falling above this range were diluted appropriately so that their A-SAA levels fell within the range of the curve. For assay validation the linearity of sample dilution was analysed by carrying out serial dilutions and the resulting data show that assay parallelism is observed in the ELISA. The reproducibility of the ELISA method was analysed by intra-assay and inter-assay variability . The intra-assay coefficient of variation from twenty replicate assays of three A-SAA serum samples (A-SAA concentrations were 5, 130 and 244 mg/L) were 4.8, 5.0 and 6.7%, respectively. The inter-assay coefficient of variation in ten replicate assays on the same serum samples were 8.0, 6.2 and 6.0%, respectively.

The normal range for A-SAA was analysed using 50 serum samples from healthy individuals, and determined to be 0.4 mg/L ± 0.57 mg/L using the standard equation: mean ± 2 SD. The A-SAA concentrations in 30 serum samples from rheumatoid arthritis patients were analysed using the ELISA procedure of Example 4 and 95% of rheumatoid arthritis patients showed an elevated level of A-SAA (Fig. 8).

* * * * *

A-SAA proteins are difficult to isolate, purify and solubilise. The production of A-SAA2 by thrombin cleavage from a GST-(A-SAA2) fusion protein in conjunction with the use of Triton X-100 for solubilisation offers a means of generating large amounts of homogeneous A-SAA. As the recovery of soluble A-SAA2 by this method is possible without the use of harsh denaturants the resulting

material may be particularly suited to future studies of A-SAA structure and biological function. Antibodies generated against recombinant A-SAA2 were shown to be specific for A-SAAs and used to develop an ELISA for quantifying A-SAA in patient serum as hereinbefore exemplified. Monitoring acute phase protein levels is of considerable clinical importance in the assessment of inflammatory disease activity and response to therapy and the ELISA reported here provides a simple, rapid and reproducible method for such monitoring.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: The Provost, Fellows and Scholars...(Trinity College Dublin)
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(E) COUNTRY: Ireland
(F) POSTAL CODE (ZIP): none

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(D) STATE: County Wicklow
(E) COUNTRY: Ireland
(F) POSTAL CODE (ZIP): none

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(C) CITY: Delgany
(D) STATE: County Wicklow
(E) COUNTRY: Ireland
(F) POSTAL CODE (ZIP): none

(ii) TITLE OF INVENTION: Method for the quantitative measurement of human acute phase serum amyloid A protein

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IE S950553
(B) FILING DATE: 21-JUL-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGGGATCCGG GCGAAGCTTC TTTTCGTTC 29

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CGGAATTCAG TATTTCTCAG GCAGGCC 27

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Gly Ser Gly Arg Ser Phe Phe Ser Phe Leu Gly Glu Ala Phe Asp Gly
1               5                  10                  15

Ala Arg Asp
```

Claims:-

1. A method for the quantitative determination of human acute phase serum amyloid A protein (A-SAA), which comprises contacting a sample of a biological fluid with antibody specific for A-SAA, said sample being reacted with an organic solvent prior to or simultaneous with antibody contact.

2. A method according to Claim 1, wherein the biological fluid is plasma or serum.

3. A method according to Claim 1 or 2, wherein the antigen used to raise the anti-A-SAA is recombinant A-SAA.

4. A method according to Claim 3, wherein the antigen used to raise the anti-A-SAA is recombinant A-SAA2.

5. A method according to any preceding claim, wherein the organic solvent is a polar organic solvent.

6. A method according to Claim 5, wherein the organic solvent is a C_1-C_4 alcohol.

7. A method according to Claim 6, wherein the organic solvent includes an amount of a C_1-C_4 ether.

8. A method according to any preceding claim, wherein the organic solvent is used in an amount of 10-50% v/v of the sample diluent.

9. A method according to Claim 8, wherein the organic solvent is used in an amount of 20-30% v/v of the sample diluent.

10. A method according to any preceding claim, wherein the antibody is used on the solid phase and as a component of the detection system of an enzyme linked immunosorbant assay.

11. A test kit or pack for carrying out a method according to any one of Claims 1-10.

12. Substantially pure recombinant A-SAA2.

13. Antibody specific for A-SAA1 and A-SAA2.

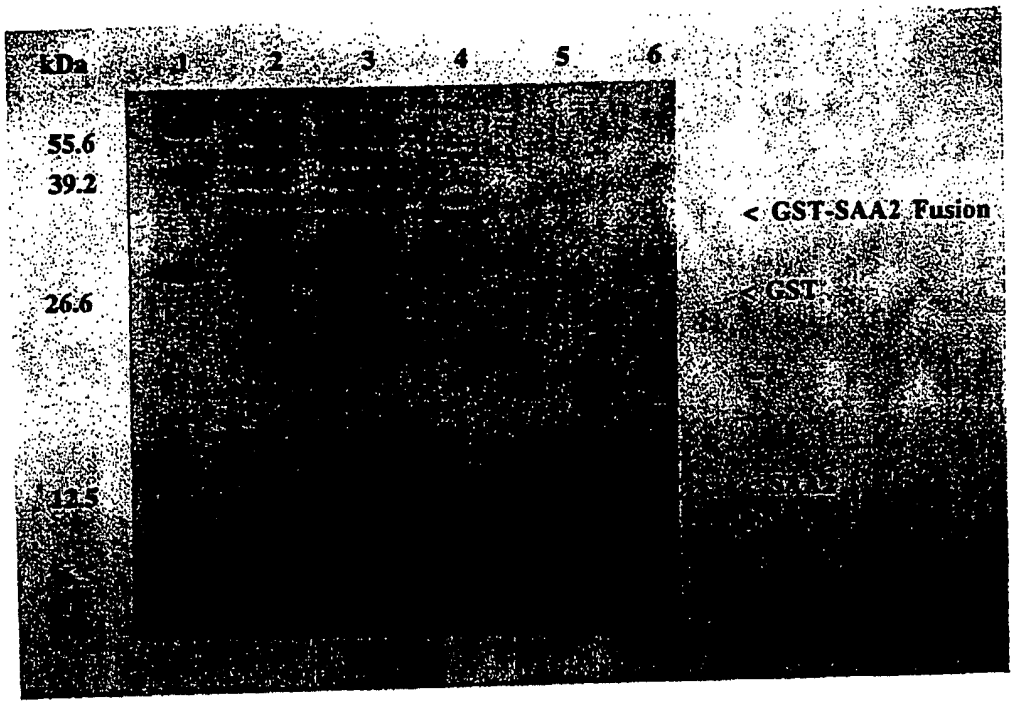

FIG. 1

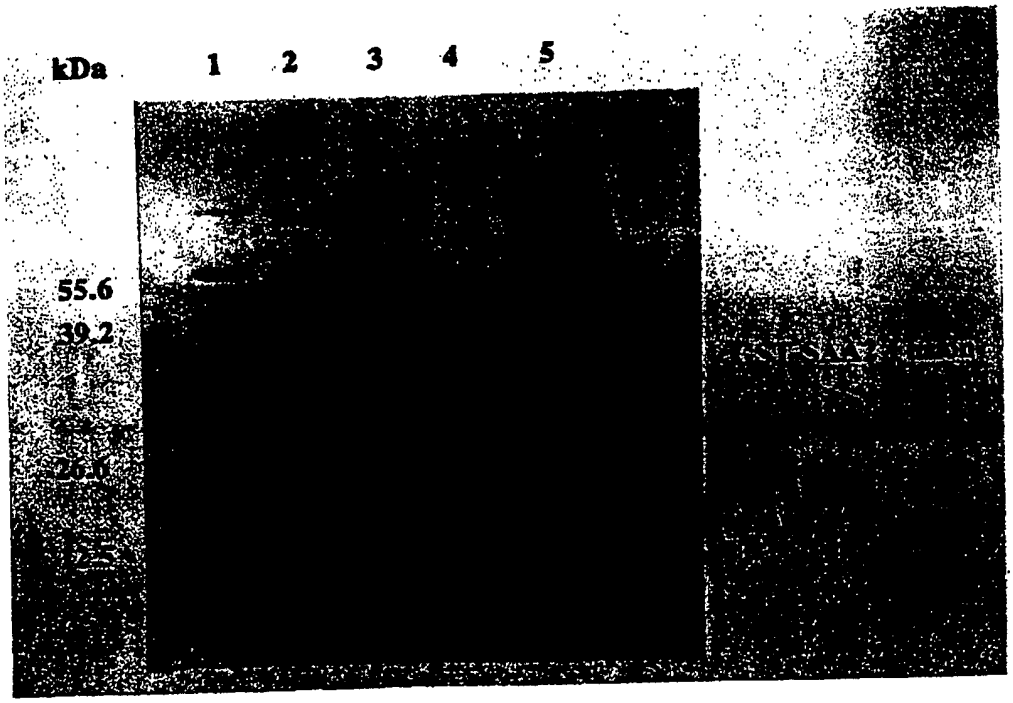

FIG. 2

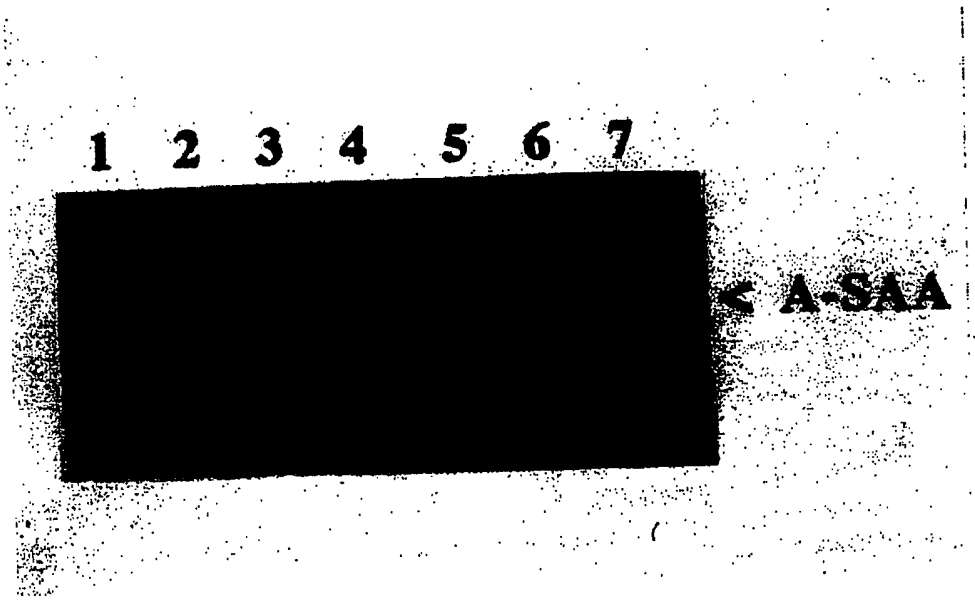

FIG. 3

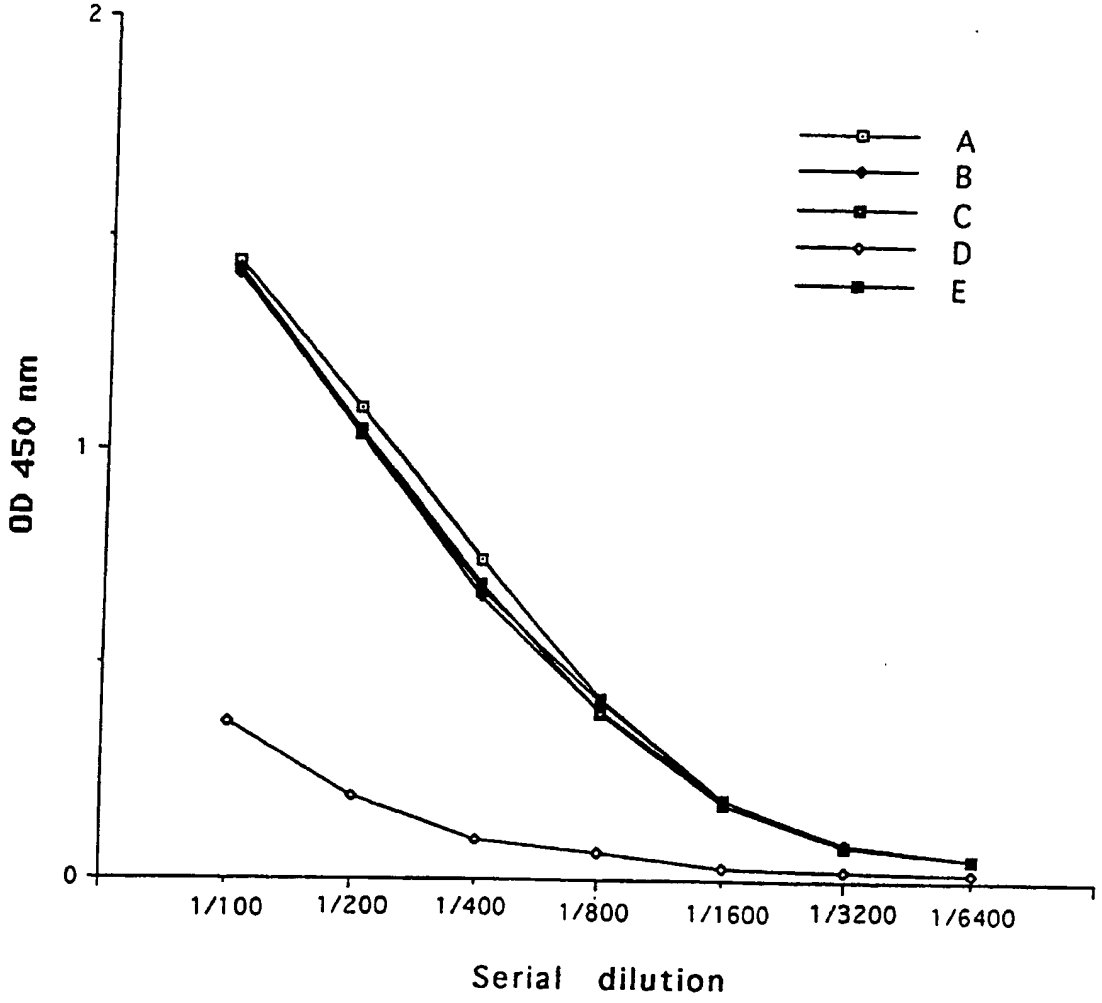

Fig. 4

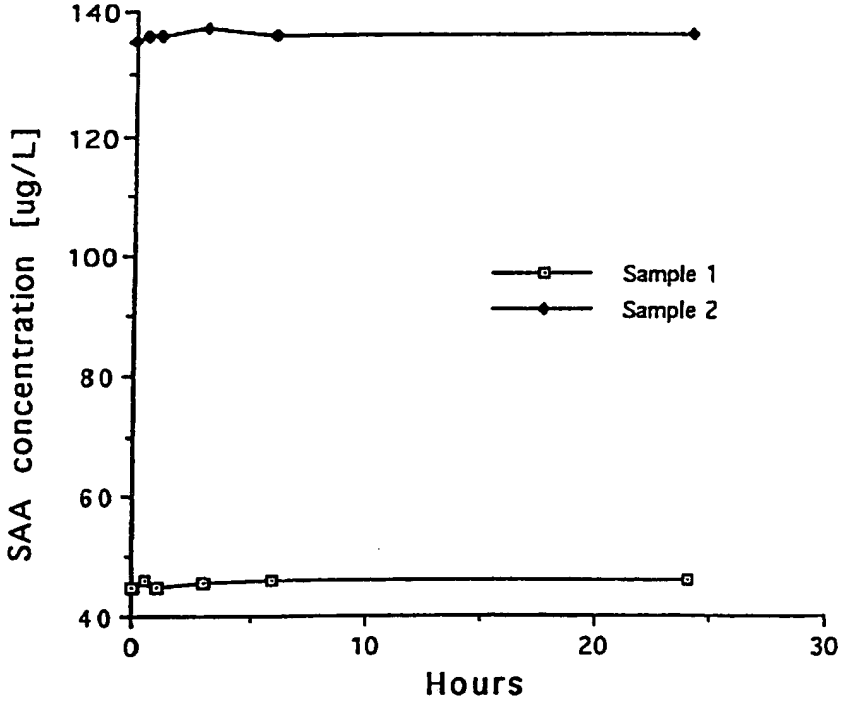
140
120
100
80
60
40
SAA concentration [ug/L]
0
10
20
30
Hours
Sample 1
Sample 2

Fig. 5

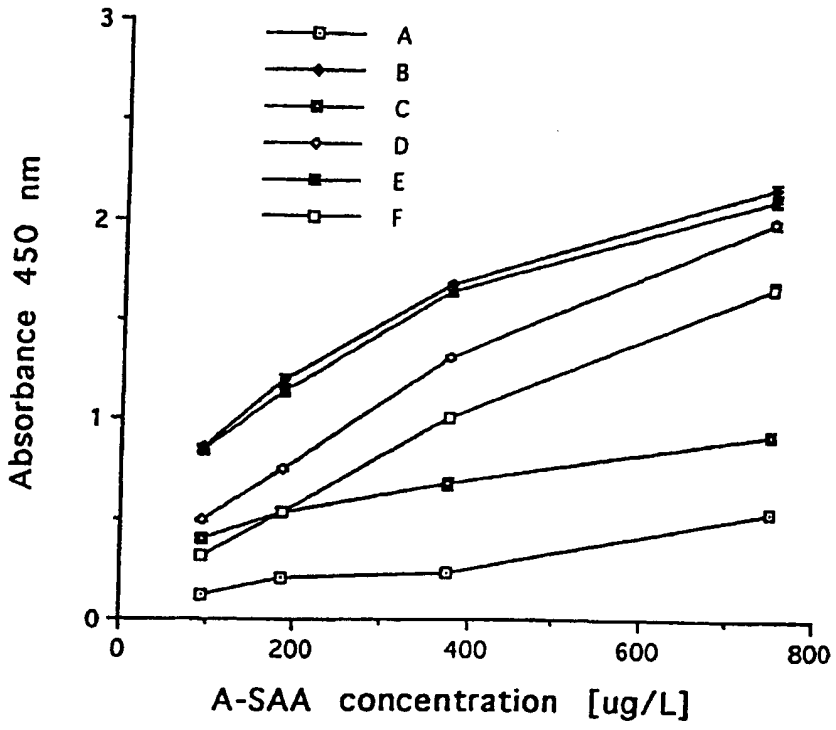
A
B
C
D
E
F
Absorbance 450 nm
0
1
2
3
0
200
400
600
800
A-SAA concentration [ug/L]

Fig. 6

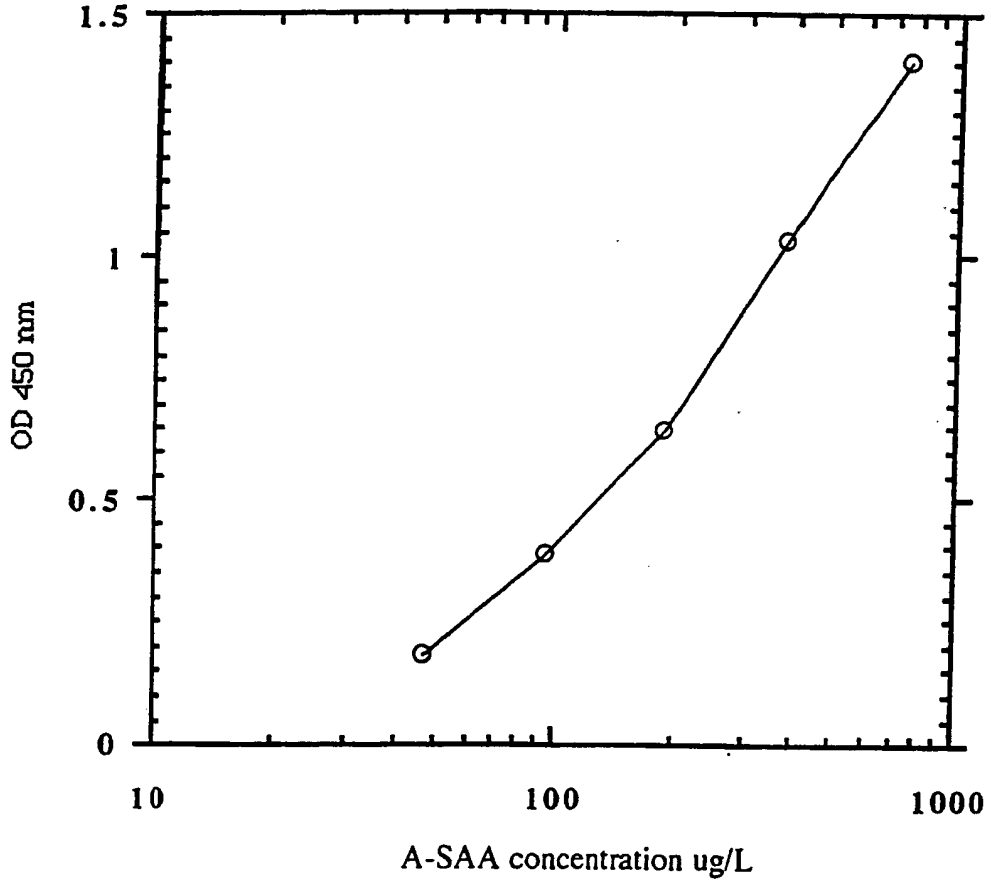

Fig. 7

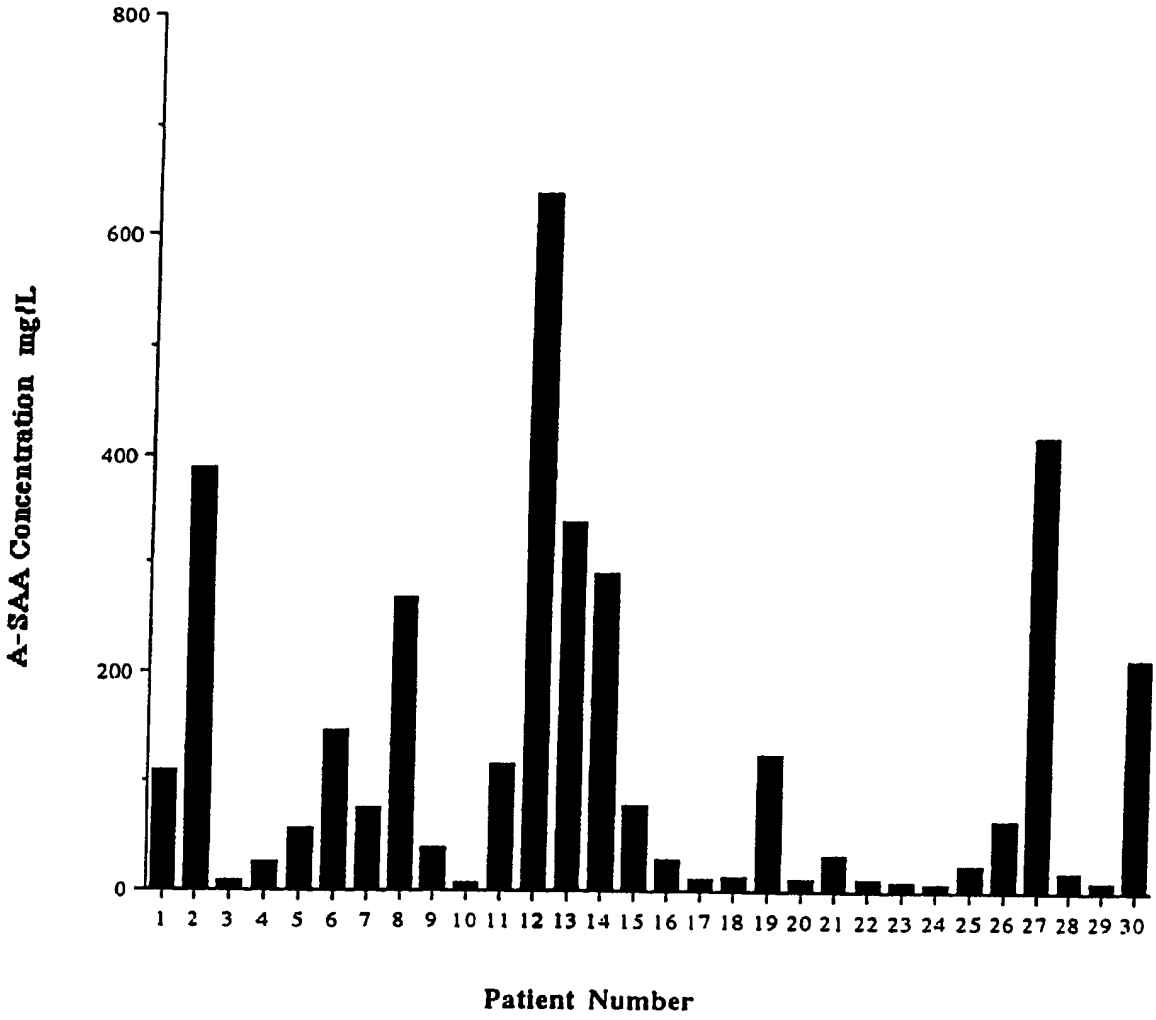
800
600
400
200
0
A-SAA Concentration mg/L
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Patient Number

Fig. 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IE 96/00042

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C07K14/47 C12N15/12 C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,91 05874 (UNIV BOSTON) 2 May 1991 see page 3, line 1 - line 10 ---	1,2,11 3-10
X	DATABASE WPI Derwent Publications Ltd., London, GB; AN 88-094820 XP002019829 & JP,A,63 044 895 (KYOWA HAKKO KOGYO KK) , 25 February 1988	12,13
Y	see abstract --- -/--	3-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 December 1996	19.12.1996
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	**Authorized officer** Wells, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHEMICAL ABSTRACTS, vol. 125, no. 15, 7 October 1996 Columbus, Ohio, US; abstract no. 190003, COSMO SOGO KENKYUSHO: "Immunoassay for serum amyloid A after dilution with hydrophilic alcohols" XP002019828 &JP-A-8178921 12/7/96 see abstract ---	1,2,5,6, 8-11
X	BIOCHEMISTRY, vol. 11, 1972, pages 2934-2938, XP002019827 HERMODSON ET AL: "Amino Acid Sequence of monkey Amyloid protein A" see the whole document -----	13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IE 96/00042

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9105874	02-05-91	US-A-	5262303	16-11-93
		EP-A-	0637340	08-02-95
		JP-T-	5501157	04-03-93
		CA-A-	2052316	11-04-92
		US-A-	5536640	16-07-96